Evidence of a Causal Effect of Estradiol on Fracture Risk in Men

Maria Nethander,[1, 2] Liesbeth Vandenput,[1] Anna L. Eriksson,[1] Sara Windahl,[1] Thomas Funck-Brentano,[1] Claes Ohlsson[1]

[1]Centre for Bone and Arthritis Research, Department of Internal Medicine and Clinical Nutrition, Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

[2]Bioinformatics Core Facility, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

Short title: Causal effect of estradiol on fracture risk in men

Keywords: bone mineral density – estradiol – testosterone – fractures – Mendelian randomization

Funding: This work was supported by the Swedish Research Council, the Swedish Foundation for Strategic Research, the ALF research grant in Gothenburg, the Lundberg Foundation, Knut and Alice Wallenberg Foundation, the Torsten and Ragnar Söderberg's Foundation and the Novo Nordisk Foundation.

Disclosure statement: The authors have nothing to disclose.

***Corresponding author and person to whom reprint requests should be made*:**

Claes Ohlsson, MD, PhD, Professor

Centre for Bone and Arthritis Research, Department of Internal Medicine and Clinical Nutrition, Institute of Medicine, Sahlgrenska Academy, University of Gothenburg

Vita Stråket 11, SE-413 45 Gothenburg, Sweden

Phone (office): +46 31 3422873, E-mail: claes.ohlsson@medic.gu.se

ABSTRACT

Context: Observational studies indicate that serum estradiol (E2) is more strongly associated with bone mineral density (BMD) than serum testosterone (T) while both E2 and T associate with fracture risk in men.

Objective: To evaluate the possible causal effect of serum E2 and T on fracture risk in men.

Design, Setting, and Participants: A Mendelian Randomization (MR) approach was undertaken using individual-level data of genotypes, BMD as estimated by quantitative ultrasound of the heel (eBMD), fractures (n=17,650), and relevant covariates of 175,583 unrelated men of European origin from the UK Biobank. The genetic instruments for serum E2 and T were taken from the most recent large scale GWAS meta-analyses on these hormones in men.

Results: MR analyses demonstrated a causal effect of serum E2 on eBMD and fracture risk. A 1 SD (or 9.6 pg/ml) genetically instrumented decrease in serum E2 was associated with a 0.38 SD decrease in eBMD (p-value 9.7×10^{-74}) and an increased risk of any fracture (OR 1.35, 95% CI, 1.18-1.55), non-vertebral major osteoporotic fractures (OR 1.75, 95% CI, 1.35-2.27) and wrist fractures (OR 2.27, 95% CI, 1.62-3.16). These causal effects of serum E2 on fracture risk were robust in sensitivity analyses and remained unchanged in stratified analyses for age, BMI, eBMD, smoking status, and physical activity. MR analyses revealed no evidence of a causal effect of T levels on fracture risk.

Conclusion: Our findings provide the first evidence of a robust causal effect of serum E2, but not T, on fracture risk in men.

PRECIS

Using a Mendelian randomization approach, we demonstrate a robust causal effect of serum estradiol, but not testosterone, on fracture risk in 175,583 unrelated European men from the UK Biobank.

INTRODUCTION

It is well recognized that sex steroids are crucial for male bone health (1). Many experimental animal studies as well as both human observational association studies and intervention studies provide evidence of a major role of estrogens over androgens on the maintenance of bone mass and strength in males (2-4). More specifically, serum estradiol (E2) correlates better than serum testosterone (T) with bone mineral density (BMD) at various sites while both low E2 and low T associate with increased fracture risk in men (1, 3, 5-10). However, the possible causal effect of E2 and T levels on fracture risk in men is unknown.

The principles of Mendelian randomization (MR) can be applied to test the role of biomarkers in disease etiology (11). MR uses genetic data to ascertain whether a given biomarker, such as E2 or T, is implicated in disease etiology, relying on a simple tenet: if a biomarker is etiologically involved in a disease process, then the genetic factors that influence the biomarker will influence disease risk (12). This established technique greatly limits confounding, since genotypes are expected to be randomly assorted at conception; further, it is free of reverse causation since genotypes are always assigned prior to the onset of disease. Thus, MR studies overcome some of the limitations of observational studies and are conceptually similar to randomized controlled trials (RCTs), but provide a lifelong assessment of exposure to a biomarker, such as low serum E2 or T levels. Furthermore, recent advances in genotyping enable the application of the MR methodology in sample sizes that are not realistic for RCTs of T or E2 therapy in men. A recent medium-sized RCT (n=211) demonstrated that a 1-year treatment with T, augmenting both serum T and E2 levels, increased volumetric bone density and estimated bone strength in older men with low serum T

(13). However, that study was underpowered for fracture analyses and could not separate causal effects of serum E2 and T on bone parameters.

We recently performed the first large scale genome-wide association study (GWAS) meta-analysis on serum E2 and identified genome-wide significant single-nucleotide polymorphisms (SNPs) associated with E2 levels in men (14). Subsequently, we used these SNPs in a 2-sample MR analysis using summary statistics from large scale GWAS meta-analyses on lumbar spine BMD and femoral neck BMD(15) and demonstrated, for the first time, a causal effect of serum E2 on BMD in men (14). However, individual-level data was not available in that study, precluding stratified analyses and evaluations of possible interactions with relevant covariates. In addition, we could not determine the causal effect of E2 on fracture risk. No previous MR analysis of the possible causal effect of serum T on BMD or fracture risk has been performed.

In the present study, we adopted an MR design to estimate the effect of genetically lowered E2 or T levels on estimated BMD (eBMD) and fracture risk in a 2-sample MR analysis using individual-level data from unrelated men of European origin in the UK Biobank (n=175,583, including 17,650 fractures), the largest male cohort worldwide. The MR instruments for serum E2 and T were the lead SNPs from the most recent and largest GWAS meta-analyses on these hormones in men of European descent (14, 16, 17).

METHODS

Study Population

UK Biobank: In 2006–2010, the UK Biobank recruited 502,647 individuals aged 37–76 years from across the UK (18). All participants provided information regarding their health and lifestyle via touch screen questionnaires, consented to physical measurements, and agreed to have their health followed. They also provided blood for future analysis. UK Biobank has ethical approval from the Northwest Multi-centre Research Ethics Committee, and informed consent was obtained from all participants. For the present study, individual participant level data of E2-associated and T-associated SNPs and evaluated phenotypes (eBMD using ultrasound of the heel and self-reported fractures) and relevant covariates (age, body mass index (BMI), smoking status, and physical activity) were available for 175,583 unrelated men of European origin (Table 1). As physical activity parameter, we used vigorous physical activity (yes/no) as it is the dichotomous physical activity parameter that was most robustly associated with eBMD (14, 16, 17).

Estimated BMD using ultrasound

Quantitative ultrasound of the heel was used to obtain a non-invasive estimate of BMD that predicts fracture (19, 20). A Sahara Clinical Bone Sonometer (Hologic Corporation, Bedford, Massachusetts, USA) was used for quantitative ultrasound assessment of calcanei in UK Biobank participants. Details of the complete protocol are publicly available on the UK Biobank website (http://www.ukbiobank.ac.uk/). eBMD (g/cm^2) was derived as a linear combination of speed of sound (SOS) and bone ultrasound attenuation (BUA) (eBMD = $0.0025926 \times (BUA + SOS) - 3.687$) (20).

Fractures

To achieve maximal power in the fracture analyses, the primary analysis was the causal effect of E2 and T on all self-reported fractures (n=17,650). Sub-analyses according to fracture type were also performed for non-vertebral major osteoporotic fractures (n= 4,379; wrist, arm, and hip) and wrist fractures (n=2,637) while the low number of hip fractures (n=341) and spine (n=418) precluded separate hip and spine fracture analyses (Table 1).

Population stratification

To reduce the risk of influence of population stratification, we only used a subset of unrelated men of European origin in the UK biobank. Pairs of individuals up to 3rd degree relatives were identified using the estimated kinship coefficients from KING's robust estimator (21).

SNP selection and data sources

We selected the lead SNPs from identified loci associated with E2 (two independent SNPs from the base model) (14) or T (three independent SNPs) (16, 17) and the estimates of their effects from the most recent and largest GWAS meta-analyses on these hormones in men.

SNP validation

To validate the selected E2 and T SNPs as instruments for our MR analysis, we evaluated them for the three MR assumptions: strong association with the exposure (E2 or T); absence of association with known confounders of the exposure-outcome association; and absence of pleiotropy, where the genetic variant influences eBMD or fracture risk through mechanisms that are independent of E2 or T. Because of randomization of alleles at conception, confounding is greatly minimized in MR studies; however, we examined if E2 or T SNPs may influence important known confounders (age, physical activity, smoking status, or BMI;

Supplemental Table 1) that may link E2 or T to BMD and/or fracture risk (4, 22-28). Pleiotropy may bias results if the chosen SNPs exert effects on eBMD or fracture risk independently of E2 or T. In this study, pleiotropy is less likely since all E2 and T associated SNPs map to genes implicated in sex steroid physiology.

MR estimates

We assessed the effects of the SNPs upon eBMD and fractures, weighting the effect of each SNP by the magnitude of its effect upon E2 or T levels. In the absence of available data on E2 or T levels in the UK Biobank, we used effect estimates from the most recent GWAS studies as instrumental variable estimates of genetically determined E2 and T in a 2-sample MR approach (29). To provide a summary measure for the effect including all genome-wide significant SNPs for E2 or T, we combined weighted estimates using fixed effects models in inverse variance weighted (IVW) MR analyses. The effect size for the meta-analysis is reported as the effect of a standard deviation (SD) change in E2 (1 SD = 9.6 pg/ml from the Framingham Heart Study) (14) or T (1 SD = 176 ng/dl from MrOS Sweden) (17), since this metric is more interpretable than an arbitrary difference (12). We also undertook power calculations to test whether our study was adequately powered to detect a clinically relevant change in the fracture outcomes (30).

Power calculations

Our E2 MR analyses had a power of 100, 72, and 25% to detect an OR of 1.4, 1.2, and 1.1, respectively, for a 1 SD change in E2 on any fracture (n=175,583; 17,650 fractures; setting alpha to 0.05). Our E2 MR analyses, excluding rs5934505 with an effect on both serum E2 and T, had a power of 100, 66, and 22% to detect an OR of 1.4, 1.2, and 1.1, respectively, for a 1 SD change in E2 on any fracture. Our T MR analyses had a power of 100, 100, and 70%

to detect an OR of 1.4, 1.2, and 1.1, respectively, for a 1 SD change in T on any fracture. Our T MR analyses, excluding rs5934505, had a power of 100, 99, and 63% to detect an OR of 1.4, 1.2, and 1.1, respectively, for a 1 SD change in T on any fracture.

Data availability

The genetic and phenotypic UK Biobank data are available upon application to the UK Biobank (https://www.ukbiobank.ac.uk/) to all *bona fide* researchers.

RESULTS

SNP selection

E2 SNPs: The lead SNPs from genome-wide significant independent loci from the most recent and largest GWAS meta-analysis on E2 were selected (14). These included two E2-associated SNPs: rs727479 in *CYP19A1* (aromatase, the enzyme converting T to E2) and rs5934505 in *FAM9B* (a protein expressed exclusively in the testis) (Supplemental Table 2). Importantly, rs727479 in *CYP19A1* influences serum E2 but not serum T, while rs5934505 in *FAM9B* influences both serum E2 and T in the same direction. By comparing the effects of these two SNPs, it is possible to separate the causal effects of serum E2 from those of T (17).
T SNPs: The lead SNPs from genome-wide significant independent loci from the most recent and largest GWAS meta-analyses on T were selected (16, 17). These included three T-associated SNPs: rs5934505 in *FAM9B*, rs10822186 in *JMJD1C* (a testicular protein with transcriptional regulatory functions in the development of the testis and proposed to regulate T synthesis) (31), and rs12150660 in *SHBG* (this SNP is highly correlated with a penta-nucleotide repeat, which affects SHBG expression *in vitro*) (17, 32) (Supplemental Table 3).

LD, confounding, and pleiotropy assessment

We found no evidence of LD between any of the E2 or T SNPs (all pairwise $r^2 \leq 0.01$).
In our literature search for potential confounders, BMI, physical activity, age, and smoking were identified as risk factors for low eBMD and/or fractures that have also been associated with E2 and/or T levels (4, 22-28). We found no significant association between the E2 or T SNPs and BMI, smoking status, age, or physical activity in the UK Biobank (Supplemental Table 1).

The E2-associated SNPs may also influence the risk of fractures independently of E2 through pleiotropy. Therefore, we performed sensitivity analyses excluding either the rs727479 in *CYP19A1* or rs5934505 in *FAM9B,* yielding very similar results as when both SNPs were evaluated together. Due to the limited number of SNPs as genetic instruments, it was not applicable to perform Egger regression.

Association of E2 and T SNPs with E2 and T serum levels

The associations of the two genome-wide significant E2 SNPs with E2 levels and the three T SNPs with T levels are described in Supplemental Tables 2 and 3, respectively (14, 16, 17). Importantly, each extra C allele of rs727479 in *CYP19A1* (coding for aromatase, the enzyme converting T into E2) was associated with a 0.145 SD reduction in serum E2 (β -0.145 SD E2/extra C allele; $p=8.2 \times 10^{-30}$, Supplemental Table 2), while it was not associated with serum T (β 0.022 SD T/extra C allele, p=0.14). Thus, this SNP is useful as an instrument to separate the causal effects of serum E2 from those of serum T. The proportion of the population-level variance in E2 levels explained by the two E2 SNPs and the proportion of variance explained by the three T SNPs are reflected by the F-statistics (*F-statistics* E2-SNPs 12.9-102.6, T-SNPs 53.6-338.2; Supplemental Tables 2 and 3).

Association of E2 SNPs with eBMD and fracture susceptibility

The effect estimates of the associations of the two E2-associated SNPs with eBMD and fracture susceptibility were derived using data from the UK Biobank in models adjusted for age, height and weight included as continuous parameters. The E2-decreasing alleles of both SNPs were significantly associated with reduced eBMD and increased risk of any fracture, non-vertebral major osteoporotic fractures and wrist fractures (Supplemental Table 2).

MR analysis for the association of E2 and eBMD

We next performed IVW MR analyses of the association between E2 and eBMD. In order to estimate the association of genetically lowered E2 with eBMD, we used a fixed-effects model including the two independent E2-decreasing alleles. A decrease in E2 levels by one SD (or 9.6 pg/ml) was associated with a 0.38 SD decrease in eBMD (p-value 9.7×10^{-74}, Fig. 1, Table 2). Age, BMI, smoking, and physical activity are associated with eBMD in men of the UK Biobank. The well-powered UK Biobank with individual level data available for relevant covariates made it possible to evaluate the causal effect of serum E2 on eBMD stratified by age (divided by median age), BMI (overweight yes/no), smoking status (current smoking yes/no), or physical activity (vigorous physical activity yes/no; Fig. 1). The causal effects of E2 on eBMD were robust and remained unchanged in stratified analyses by age, BMI, smoking status, and physical activity. A weighted genetic risk score for E2 (E2-GRS) was significantly associated with eBMD ($p=7.1 \times 10^{-63}$, Supplemental Table 4). No significant interaction effect on eBMD was observed between this E2-GRS and age, BMI, smoking status, or physical activity (Supplemental Table 4). We observed similar causal effects of E2 on eBMD in the different genotype groups when stratified according to the previously reported BMD-associated estrogen receptor-α (ERα) SNP rs4869742 (Fig. 1) (15). An additive, but not synergistic effect (no significant interaction), of the ERα SNP rs4869742 (15) and the E2-GRS on eBMD was observed (Fig. 1, Supplemental Table 4).

MR analysis for the association of E2 and fracture susceptibility

IVW MR analyses revealed that a decrease in E2 levels by one SD was associated with an increased risk of any fracture (OR 1.35, 95% CI, 1.18-1.55), non-vertebral major osteoporotic fractures (*wrist, arm and hip*; OR 1.75, 95% CI, 1.35-2.27), and wrist fractures (OR 2.27, 95% CI, 1.62-3.16; Fig. 2, Table 2). Sensitivity analyses excluding either the rs727479 in

CYP19A1 (with an impact on serum E2 but not T) or rs5934505 in *FAM9B* (with an impact on both E2 and T in the same direction) yielded very similar results as when both SNPs were evaluated together (Table 2). This finding indicates that E2 exerts a causal effect on fracture risk independently of serum T.

MR analyses stratified by age (divided by median age), BMI (overweight yes/no), eBMD (divided by the median), smoking status (current smoking yes/no), or physical activity (vigorous activity yes/no) revealed that the causal effect of E2 on fracture risk was robust and remained significant and of similar magnitude in stratified analyses (Fig. 3). The E2-GRS was significantly associated with fracture risk and no significant interaction effect on fracture risk was observed for this E2-GRS and age, BMI, smoking status, or physical activity (Supplemental Table 4). The causal effect of E2 on fracture risk was also similar in the different genotype groups when stratified according to ERα SNP rs4869742 (Fig. 3). An additive, but not synergistic effect (no significant interaction), of the ERα SNP rs4869742 (15) and the E2-GRS on fracture risk was observed (Fig. 3, Supplemental Table 4).

MR analysis for the association of T and fracture susceptibility

MR analyses including all three T SNPs (also rs5934505 in FAM9B with an impact on both E2 and T) indicated a modest causal effect of T on eBMD (a 0.14 SD decrease per SD decrease in T; Supplemental Table 5). Sensitivity analyses excluding possible E2-mediated effects by removing rs5934505 showed no evidence of a causal effect of T on eBMD (Supplemental Table 5). Neither MR analyses including all three T SNPs (OR 1.03, 95% CI 0.93-1.15 per SD decrease in T for any fracture) nor MR analyses excluding rs5934505 revealed any significant causal effect of T on fracture risk (Supplemental Table 5).

DISCUSSION

Both experimental animal studies and human interventional studies have revealed a major role of estrogens over androgens on the maintenance of bone mass in males (2-4). Observational studies have reported that both low serum T and low serum E2 are associated with increased fracture risk but the possible causal effect of these hormones on fracture risk in men is unknown. Using MR analyses, we, herein, provide strong evidence of a causal effect of serum E2, but not T, on fracture risk in men of European descent.

Our finding of a causal effect of E2 on eBMD in the heel confirms a recent MR report demonstrating a causal effect of serum E2 on both femoral neck and lumbar spine BMD (14). The magnitudes of the causal effects were similar: a 1 SD genetically instrumented decrease in serum E2 was associated with a 0.38 SD decrease in eBMD in the present study, and a 0.46 SD and 0.36 SD decrease in lumbar spine BMD and femoral neck BMD, respectively, in the previous MR report (14). A limitation with the previous MR report on the causal effect of E2 on DXA-derived BMD was the lack of individual-level data, precluding stratified analyses and evaluation of interactions (14). In the present study, the availability of the well-powered UK Biobank dataset with individual level data of relevant covariates enabled us to evaluate the causal effect of serum E2 on eBMD stratified by age, BMI, smoking status, or physical activity. The causal effects of E2 on eBMD were robust and remained unchanged in stratified analyses for these parameters known to influence BMD. In addition, no significant interaction effect was observed for the E2-GRS and age, BMI, smoking status, or physical activity on eBMD. Collectively, these findings demonstrate that serum E2 exerts a robust causal effect on eBMD independent of age, BMI, smoking status, and physical activity. This strong evidence of a causal effect of E2 on eBMD is in accordance with the results from a recent medium-

sized RCT (n=211) demonstrating that 1-year treatment with T, augmenting both serum T and E2 levels, increased volumetric bone density and estimated bone strength in older men with low serum T (13).

Importantly, in the present study we provide the first evidence of a robust causal effect of serum E2 on fracture risk. The magnitude of the causative effect of E2 on all fracture risk (OR 1.35 per SD decrease in E2) in the present study is very similar to the observational association of E2 with any incident fracture (hazard ratio of 1.34 per SD decrease in E2) previously reported for elderly men in the MrOS Sweden cohort (9). Interestingly, the causal effects of E2 on non-vertebral major osteoporotic fracture risk (OR 1.75 per SD decrease in E2) and wrist fractures risk (OR 2.27 per SD decrease in E2) were substantial, suggesting that serum E2 has a major impact on clinically relevant osteoporotic fractures in men. A limitation of the present study is that the low number of hip and spine fractures precluded separate hip and spine fracture analyses. The causal effects of E2 on fracture risk remained unchanged in stratified analyses for several parameters known to influence bone health including age, BMI, eBMD, smoking status, and physical activity (4, 22-28). In addition, no significant interaction effect was observed for the E2-GRS and any of these parameters on fracture risk.

Sensitivity analyses including only rs727479, with an impact on serum E2 but not serum T, yielded very similar results as when both E2 SNPs were evaluated together. This finding indicates that E2 exerts a causal effect on fracture risk independently of serum T. Thus, serum E2 exerts a robust causal effect on eBMD and fracture risk that seems to be independent of many other known bone health-associated factors. Based on the findings in the present study, one may speculate that treatments augmenting serum E2, such as T supplementation, may reduce fracture risk in men. However, to avoid androgen receptor-related side effects one

might consider bone-specific selective estrogen receptor modulators (SERMs) to reduce fracture risk in men. In addition, our findings indicate that non-aromatizable androgens, not augmenting E2, will be inefficient in reducing fracture risk in men. The finding of a causal effect of serum E2, but not T, on fracture risk might also be of importance for estimating possible skeletal side effects of endocrine therapies in men with prostate cancer. Our findings suggests that chemical castration with GnRH agonists as well as CYP17 inhibition using abiraterone will result in increased fracture risk in these patients, since both treatments will reduce serum T as well as serum E2 levels. This notion is supported by a substantially increased fracture risk in prostate cancer patients after chemical castration (4, 33). In contrast, our data, showing no causal effect of T on fracture risk, indicate that endocrine prostate cancer treatment with the specific androgen receptor antagonist enzalutamide could avoid these skeletal side-effects. Nevertheless, it should be acknowledged that the present MR analyses are based on circulating levels of sex steroids. It is well known that sex steroids also are synthesized and metabolized locally in tissues (34). The resulting local tissue levels may be important for local sex steroid action but their potential causal effects are not possible to evaluate by the MR approach used in the present study.

Experimental animal studies as well as a case of a man with a mutation in ERα demonstrate that ERα is the major ER mediating the effect of E2 on the male skeleton (4, 35, 36). The *ERα* locus is one of very few loci that have been robustly associated with both BMD and fracture risk in GWAS meta-analyses (15). We hypothesized that genetic variants in *ERα* might interact with serum E2 (the ligand) for the effect on eBMD and fracture risk. However, the causal effect of serum E2 on eBMD and fracture risk was similar in the different genotypes of the previously reported BMD and fracture associated ERα SNP rs4869742 (15). We observed an additive effect of rs4869742 and a serum E2 genetic risk score on eBMD and fracture risk

but no significant interaction effect was observed. These findings indicate that genetic variants influencing the levels of the ligand (E2) and a genetic determinant in the *ERα* locus affecting the ER responsiveness have an additive estrogenic activity in bone.

We observed no evidence of a causal effect of serum T on fracture risk in the present MR analyses. Although the present T MR analyses had 100% and 70% power to detect an OR of 1.2 and 1.1, respectively, per SD decrease in serum T for any fracture, we cannot exclude a minor causal effect of serum T on fracture risk. The lack of any clear causal effect of testosterone on fracture risk in the present MR analyses contrasts with findings from a number of clinical and experimental animal studies (4, 8, 13, 37).

Strengths of this study include the large sample size with individual level data available for SNPs used as genetic instruments, eBMD, fractures, and relevant covariates, making it possible to perform subgroup analyses. This study also has limitations. While we controlled for pleiotropy in sensitivity analyses excluding SNPs, residual bias cannot be excluded since the exact function of the SNPs studied is unknown. However, all the SNPs lie in, or near, genes well validated for their role in sex steroid physiology. The null results for T could be explained by canalization, which is a phenomenon resulting in compensatory feedback mechanisms (11). Another limitation with the present study is that serum sex steroid levels were not available in the UK Biobank. In addition, it should be noted that the GWAS, which identified the selected genetic instruments, not only used gold standard MS-based techniques (38) but also immunoassay-based techniques with a questionable specificity at the lower concentration range (39, 40) to assess sex steroid levels. Our MR analysis might also be limited in its ability to elucidate a possible causal role of biologically active T as it is tightly bound to the high-affinity binding protein SHBG. Moreover, the impact of genetic variants in the androgen receptor and their possible interactions with serum testosterone on fracture risk

were not evaluated in the present study. A further limitation with the present MR approach is that the effects of endocrine disrupters, variation in local aromatase activity in bone, and the interplay between free and bound fractions could not be evaluated. Our analyses were restricted to white populations of European ancestry, and further work will be required to investigate their relevance in populations of different ethnicity.

In conclusion, we provide the first MR evidence of a robust causal effect of serum E2, but not T, on fracture risk in men. Our findings may have direct clinical implications for prostate cancer patients when comparing the possible skeletal side-effects of endocrine treatments affecting both E2 and T levels with those only modulating androgen receptor action.

Acknowledgements

This research has been conducted using the UK Biobank Resource under Application Number 2694.

Funding

This work was supported by the Swedish Research Council, the Swedish Foundation for Strategic Research, the ALF research grant in Gothenburg, the Lundberg Foundation, Knut and Alice Wallenberg Foundation, the Torsten and Ragnar Söderberg's Foundation and the Novo Nordisk Foundation.

REFERENCES

1. Vandenput L, Ohlsson C. Estrogens as regulators of bone health in men. *Nat Rev Endocrinol.* 2009;5(8):437-443.
2. Sims NA, Clement-Lacroix P, Minet D, Fraslon-Vanhulle C, Gaillard-Kelly M, Resche-Rigon M, Baron R. A functional androgen receptor is not sufficient to allow estradiol to protect bone after gonadectomy in estradiol receptor-deficient mice. *J Clin Invest.* 2003;111(9):1319-1327.
3. Finkelstein JS, Lee H, Leder BZ, Burnett-Bowie SA, Goldstein DW, Hahn CW, Hirsch SC, Linker A, Perros N, Servais AB, Taylor AP, Webb ML, Youngner JM, Yu EW. Gonadal steroid-dependent effects on bone turnover and bone mineral density in men. *J Clin Invest.* 2016;126(3):1114-1125.
4. Vanderschueren D, Laurent MR, Claessens F, Gielen E, Lagerquist MK, Vandenput L, Borjesson AE, Ohlsson C. Sex steroid actions in male bone. *Endocr Rev.* 2014;35(6):906-960.
5. Amin S, Zhang Y, Felson DT, Sawin CT, Hannan MT, Wilson PW, Kiel DP. Estradiol, testosterone, and the risk for hip fractures in elderly men from the Framingham Study. *Am J Med.* 2006;119(5):426-433.
6. Amin S, Zhang Y, Sawin CT, Evans SR, Hannan MT, Kiel DP, Wilson PW, Felson DT. Association of hypogonadism and estradiol levels with bone mineral density in elderly men from the Framingham study. *Ann Intern Med.* 2000;133(12):951-963.
7. LeBlanc ES, Nielson CM, Marshall LM, Lapidus JA, Barrett-Connor E, Ensrud KE, Hoffman AR, Laughlin G, Ohlsson C, Orwoll ES, Osteoporotic Fractures in Men Study G. The effects of serum testosterone, estradiol, and sex hormone binding globulin levels on fracture risk in older men. *J Clin Endocrinol Metab.* 2009;94(9):3337-3346.
8. Meier C, Nguyen TV, Handelsman DJ, Schindler C, Kushnir MM, Rockwood AL, Meikle AW, Center JR, Eisman JA, Seibel MJ. Endogenous sex hormones and incident fracture risk in older men: the Dubbo Osteoporosis Epidemiology Study. *Arch Intern Med.* 2008;168(1):47-54.
9. Mellström D, Vandenput L, Mallmin H, Holmberg AH, Lorentzon M, Oden A, Johansson H, Orwoll ES, Labrie F, Karlsson MK, Ljunggren O, Ohlsson C. Older men with low serum estradiol and high serum SHBG have an increased risk of fractures. *J Bone Miner Res.* 2008;23(10):1552-1560.
10. Khosla S, Melton LJ, 3rd, Atkinson EJ, O'Fallon WM. Relationship of serum sex steroid levels to longitudinal changes in bone density in young versus elderly men. *J Clin Endocrinol Metab.* 2001;86(8):3555-3561.
11. Lawlor DA, Harbord RM, Sterne JA, Timpson N, Davey Smith G. Mendelian randomization: using genes as instruments for making causal inferences in epidemiology. *Stat Med.* 2008;27(8):1133-1163.
12. Manousaki D, Paternoster L, Standl M, Moffatt MF, Farrall M, Bouzigon E, Strachan DP, Demenais F, Lathrop M, Cookson W, Richards JB. Vitamin D levels and susceptibility to asthma, elevated immunoglobulin E levels, and atopic dermatitis: A Mendelian randomization study. *PLoS Med.* 2017;14(5):e1002294.
13. Snyder PJ, Kopperdahl DL, Stephens-Shields AJ, Ellenberg SS, Cauley JA, Ensrud KE, Lewis CE, Barrett-Connor E, Schwartz AV, Lee DC, Bhasin S, Cunningham GR, Gill TM, Matsumoto AM, Swerdloff RS, Basaria S, Diem SJ, Wang C, Hou X, Cifelli D, Dougar D, Zeldow B, Bauer DC, Keaveny TM. Effect of testosterone treatment on volumetric bone density and strength in older men with low testosterone: a controlled clinical trial. *JAMA Intern Med.* 2017;177(4):471-479.
14. Eriksson AL, Perry JRB, Coviello AD, Delgado GE, Ferrucci L, Hoffman AR, Huhtaniemi IT, Ikram MA, Karlsson MK, Kleber ME, Laughlin GA, Liu Y, Lorentzon M, Lunetta KL, Mellstrom D, Murabito JM, Murray A, Nethander M, Nielson CM, Prokopenko I, Pye SR, Raffel LJ, Rivadeneira F, Srikanth P, Stolk L, Teumer A, Travison TG, Uitterlinden AG, Vaidya D, Vanderschueren D, Zmuda JM, Marz W, Orwoll ES, Ouyang P, Vandenput L, Wu FCW, de Jong FH, Bhasin S, Kiel DP, Ohlsson C. Genetic determinants of circulating estrogen levels, and

evidence of a causal Effect of estradiol on bone density in men. *J Clin Endocrinol Metab.* 2018;

15. Estrada K, Styrkarsdottir U, Evangelou E, Hsu YH, Duncan EL, Ntzani EE, Oei L, Albagha OM, Amin N, Kemp JP, Koller DL, Li G, Liu CT, Minster RL, Moayyeri A, Vandenput L, Willner D, Xiao SM, Yerges-Armstrong LM, Zheng HF, Alonso N, Eriksson J, Kammerer CM, Kaptoge SK, Leo PJ, Thorleifsson G, Wilson SG, Wilson JF, Aalto V, Alen M, Aragaki AK, Aspelund T, Center JR, Dailiana Z, Duggan DJ, Garcia M, Garcia-Giralt N, Giroux S, Hallmans G, Hocking LJ, Husted LB, Jameson KA, Khusainova R, Kim GS, Kooperberg C, Koromila T, Kruk M, Laaksonen M, Lacroix AZ, Lee SH, Leung PC, Lewis JR, Masi L, Mencej-Bedrac S, Nguyen TV, Nogues X, Patel MS, Prezelj J, Rose LM, Scollen S, Siggeirsdottir K, Smith AV, Svensson O, Trompet S, Trummer O, van Schoor NM, Woo J, Zhu K, Balcells S, Brandi ML, Buckley BM, Cheng S, Christiansen C, Cooper C, Dedoussis G, Ford I, Frost M, Goltzman D, Gonzalez-Macias J, Kahonen M, Karlsson M, Khusnutdinova E, Koh JM, Kollia P, Langdahl BL, Leslie WD, Lips P, Ljunggren O, Lorenc RS, Marc J, Mellstrom D, Obermayer-Pietsch B, Olmos JM, Pettersson-Kymmer U, Reid DM, Riancho JA, Ridker PM, Rousseau F, Slagboom PE, Tang NL, Urreizti R, Van Hul W, Viikari J, Zarrabeitia MT, Aulchenko YS, Castano-Betancourt M, Grundberg E, Herrera L, Ingvarsson T, Johannsdottir H, Kwan T, Li R, Luben R, Medina-Gomez C, Palsson ST, Reppe S, Rotter JI, Sigurdsson G, van Meurs JB, Verlaan D, Williams FM, Wood AR, Zhou Y, Gautvik KM, Pastinen T, Raychaudhuri S, Cauley JA, Chasman DI, Clark GR, Cummings SR, Danoy P, Dennison EM, Eastell R, Eisman JA, Gudnason V, Hofman A, Jackson RD, Jones G, Jukema JW, Khaw KT, Lehtimaki T, Liu Y, Lorentzon M, McCloskey E, Mitchell BD, Nandakumar K, Nicholson GC, Oostra BA, Peacock M, Pols HA, Prince RL, Raitakari O, Reid IR, Robbins J, Sambrook PN, Sham PC, Shuldiner AR, Tylavsky FA, van Duijn CM, Wareham NJ, Cupples LA, Econs MJ, Evans DM, Harris TB, Kung AW, Psaty BM, Reeve J, Spector TD, Streeten EA, Zillikens MC, Thorsteinsdottir U, Ohlsson C, Karasik D, Richards JB, Brown MA, Stefansson K, Uitterlinden AG, Ralston SH, Ioannidis JP, Kiel DP, Rivadeneira F. Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. *Nat Genet.* 2012;44(5):491-501.
16. Jin G, Sun J, Kim ST, Feng J, Wang Z, Tao S, Chen Z, Purcell L, Smith S, Isaacs WB, Rittmaster RS, Zheng SL, Condreay LD, Xu J. Genome-wide association study identifies a new locus JMJD1C at 10q21 that may influence serum androgen levels in men. *Hum Mol Genet.* 2012;21(23):5222-5228.
17. Ohlsson C, Wallaschofski H, Lunetta KL, Stolk L, Perry JR, Koster A, Petersen AK, Eriksson J, Lehtimaki T, Huhtaniemi IT, Hammond GL, Maggio M, Coviello AD, Group ES, Ferrucci L, Heier M, Hofman A, Holliday KL, Jansson JO, Kahonen M, Karasik D, Karlsson MK, Kiel DP, Liu Y, Ljunggren O, Lorentzon M, Lyytikainen LP, Meitinger T, Mellstrom D, Melzer D, Miljkovic I, Nauck M, Nilsson M, Penninx B, Pye SR, Vasan RS, Reincke M, Rivadeneira F, Tajar A, Teumer A, Uitterlinden AG, Ulloor J, Viikari J, Volker U, Volzke H, Wichmann HE, Wu TS, Zhuang WV, Ziv E, Wu FC, Raitakari O, Eriksson A, Bidlingmaier M, Harris TB, Murray A, de Jong FH, Murabito JM, Bhasin S, Vandenput L, Haring R. Genetic determinants of serum testosterone concentrations in men. *PLoS Genet.* 2011;7(10):e1002313.
18. Collins R. What makes UK Biobank special? *Lancet.* 2012;379(9822):1173-1174.
19. Bauer DC, Gluer CC, Cauley JA, Vogt TM, Ensrud KE, Genant HK, Black DM. Broadband ultrasound attenuation predicts fractures strongly and independently of densitometry in older women. A prospective study. Study of Osteoporotic Fractures Research Group. *Arch Intern Med.* 1997;157(6):629-634.
20. Kemp JP, Morris JA, Medina-Gomez C, Forgetta V, Warrington NM, Youlten SE, Zheng J, Gregson CL, Grundberg E, Trajanoska K, Logan JG, Pollard AS, Sparkes PC, Ghirardello EJ, Allen R, Leitch VD, Butterfield NC, Komla-Ebri D, Adoum AT, Curry KF, White JK, Kussy F, Greenlaw KM, Xu C, Harvey NC, Cooper C, Adams DJ, Greenwood CMT, Maurano MT, Kaptoge S, Rivadeneira F, Tobias JH, Croucher PI, Ackert-Bicknell CL, Bassett JHD, Williams GR, Richards JB, Evans DM. Identification of 153 new loci associated with heel bone mineral

density and functional involvement of GPC6 in osteoporosis. *Nat Genet.* 2017;49(10):1468-1475.
21. Manichaikul A, Mychaleckyj JC, Rich SS, Daly K, Sale M, Chen WM. Robust relationship inference in genome-wide association studies. *Bioinformatics.* 2010;26(22):2867-2873.
22. Cunningham GR, Stephens-Shields AJ, Rosen RC, Wang C, Ellenberg SS, Matsumoto AM, Bhasin S, Molitch ME, Farrar JT, Cella D, Barrett-Connor E, Cauley JA, Cifelli D, Crandall JP, Ensrud KE, Fluharty L, Gill TM, Lewis CE, Pahor M, Resnick SM, Storer TW, Swerdloff RS, Anton S, Basaria S, Diem S, Tabatabaie V, Hou X, Snyder PJ. Association of sex hormones with sexual function, vitality, and physical function of symptomatic older men with low testosterone levels at baseline in the testosterone trials. *J Clin Endocrinol Metab.* 2015;100(3):1146-1155.
23. Kanis JA, Johnell O, Oden A, Johansson H, McCloskey E. FRAX and the assessment of fracture probability in men and women from the UK. *Osteoporos Int.* 2008;19(4):385-397.
24. Kaufman JM, Vermeulen A. The decline of androgen levels in elderly men and its clinical and therapeutic implications. *Endocr Rev.* 2005;26(6):833-876.
25. Lorentzon M, Mellstrom D, Ohlsson C. Association of amount of physical activity with cortical bone size and trabecular volumetric BMD in young adult men: the GOOD study. *J Bone Miner Res.* 2005;20(11):1936-1943.
26. Swiecicka A, Lunt M, Ahern T, O'Neill TW, Bartfai G, Casanueva FF, Forti G, Giwercman A, Han TS, Lean MEJ, Pendleton N, Punab M, Slowikowska-Hilczer J, Vanderschueren D, Huhtaniemi IT, Wu FCW, Rutter MK, Group ES. Nonandrogenic anabolic hormones predict risk of frailty: European Male Ageing Study prospective data. *J Clin Endocrinol Metab.* 2017;102(8):2798-2806.
27. Tajar A, Forti G, O'Neill TW, Lee DM, Silman AJ, Finn JD, Bartfai G, Boonen S, Casanueva FF, Giwercman A, Han TS, Kula K, Labrie F, Lean ME, Pendleton N, Punab M, Vanderschueren D, Huhtaniemi IT, Wu FC, Group E. Characteristics of secondary, primary, and compensated hypogonadism in aging men: evidence from the European Male Ageing Study. *J Clin Endocrinol Metab.* 2010;95(4):1810-1818.
28. Wu FC, Tajar A, Beynon JM, Pye SR, Silman AJ, Finn JD, O'Neill TW, Bartfai G, Casanueva FF, Forti G, Giwercman A, Han TS, Kula K, Lean ME, Pendleton N, Punab M, Boonen S, Vanderschueren D, Labrie F, Huhtaniemi IT, Group E. Identification of late-onset hypogonadism in middle-aged and elderly men. *N Engl J Med.* 2010;363(2):123-135.
29. Burgess S, Butterworth A, Thompson SG. Mendelian randomization analysis with multiple genetic variants using summarized data. *Genet Epidemiol.* 2013;37(7):658-665.
30. Brion MJ, Shakhbazov K, Visscher PM. Calculating statistical power in Mendelian randomization studies. *Int J Epidemiol.* 2013;42(5):1497-1501.
31. Kim SM, Kim JY, Choe NW, Cho IH, Kim JR, Kim DW, Seol JE, Lee SE, Kook H, Nam KI, Kook H, Bhak YY, Seo SB. Regulation of mouse steroidogenesis by WHISTLE and JMJD1C through histone methylation balance. *Nucleic Acids Res.* 2010;38(19):6389-6403.
32. Hogeveen KN, Talikka M, Hammond GL. Human sex hormone-binding globulin promoter activity is influenced by a (TAAAA)n repeat element within an Alu sequence. *J Biol Chem.* 2001;276(39):36383-36390.
33. Shahinian VB, Kuo YF, Freeman JL, Goodwin JS. Risk of fracture after androgen deprivation for prostate cancer. *N Engl J Med.* 2005;352(2):154-164.
34. Labrie F, Luu-The V, Labrie C, Belanger A, Simard J, Lin SX, Pelletier G. Endocrine and intracrine sources of androgens in women: inhibition of breast cancer and other roles of androgens and their precursor dehydroepiandrosterone. *Endocr Rev.* 2003;24(2):152-182.
35. Ohlsson C, Vandenput L. The role of estrogens for male bone health. *Eur J Endocrinol.* 2009;160(6):883-889.
36. Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS. Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. *N Engl J Med.* 1994;331(16):1056-1061.

37. Kawano H, Sato T, Yamada T, Matsumoto T, Sekine K, Watanabe T, Nakamura T, Fukuda T, Yoshimura K, Yoshizawa T, Aihara KI, Yamamoto Y, Nakamichi Y, Metzger D, Chambon P, Nakamura K, Kawaguchi H, Kato S. Suppressive function of androgen receptor in bone resorption. *Proc Natl Acad Sci USA.* 2003;100(16):9416-9421.
38. Rosner W, Hankinson SE, Sluss PM, Vesper HW, Wierman ME. Challenges to the measurement of estradiol: an endocrine society position statement. *J Clin Endocrinol Metab.* 2013;98(4):1376-1387.
39. Lee JS, Ettinger B, Stanczyk FZ, Vittinghoff E, Hanes V, Cauley JA, Chandler W, Settlage J, Beattie MS, Folkerd E, Dowsett M, Grady D, Cummings SR. Comparison of methods to measure low serum estradiol levels in postmenopausal women. *J Clin Endocrinol Metab.* 2006;91(10):3791-3797.
40. Huhtaniemi IT, Tajar A, Lee DM, O'Neill TW, Finn JD, Bartfai G, Boonen S, Casanueva FF, Giwercman A, Han TS, Kula K, Labrie F, Lean ME, Pendleton N, Punab M, Silman AJ, Vanderschueren D, Forti G, Wu FC. Comparison of serum testosterone and estradiol measurements in 3174 European men using platform immunoassay and mass spectrometry; relevance for the diagnostics in aging men. *Eur J Endocrinol.* 2012;166(6):983-991.

Figure legends

Figure 1. Stratified analyses of the estimated causal effect of estradiol (E2) on eBMD. Betas for eBMD are given as SD eBMD per SD decrease in E2 (95% confidence intervals). eBMD = estimated BMD in the heel using ultrasound. ER = estrogen receptor.

Figure 2. Estimated causal effect of estradiol (E2) on fracture risk. Odds ratios for fracture risk are given per SD decrease in E2 (95% confidence intervals). Total numbers of subjects/fracture cases are given within brackets.

Figure 3. Stratified analyses of the estimated causal effect of estradiol (E2) on any fracture. Odds ratios for fracture risk are given per SD decrease in E2 (95% confidence intervals). Total numbers of subjects/fracture cases are given within brackets. eBMD = estimated BMD in the heel using ultrasound. ER = estrogen receptor.

Table 1. Baseline Characteristics of the Study Participants	
	n=175,583
Age (years)	56.9 (8.1)
Weight (kg)	86.2 (14.3)
Height (cm)	175.9 (6.8)
BMI (kg/m^2)	27.8 (4.2)
Current smoking (n, %)	21,597 (12.2)
Vigorous physical activity (n, %)	98,967 (56.0)
eBMD (g/cm^2)	0.56 (0.12)
All fractures (n,%)	17,650 (10.0)
Non-vertebral major osteoporotic fractures (n,%)	4,379 (2.5)
Wrist fractures (n,%)	2,637 (1.5)

Values are given as mean (SD) or n (%). Non-vertebral major osteoporotic fractures are defined as wrist, arm, or hip fractures. eBMD = estimated BMD in the heel using ultrasound.

Table 2. Estimated Causal Effect of E2 on eBMD and Fractures

							eBMD			All fractures			Non-vertebral major osteoporotic fractures			Wrist fractures		
Genetic instrument	**Chr**	**Position**	**EA**	**OA**	**Freq EA**	**Gene**	**Beta**	**SE**	**P**	**OR**	**95% CI**	**P**	**OR**	**95% CI**	**P**	**OR**	**95% CI**	**P**
rs5934505	23	8913826	T	C	0.73	FAM9B	-0.51	0.04	1.9E-38	1.34	(1.04, 1.73)	2.4E-02	2.36	(1.43, 3.90)	7.6E-04	2.85	(1.49, 5.44)	1.5E-03
rs727479	15	51534547	C	A	0.35	CYP19	-0.33	0.02	2.4E-40	1.36	(1.16, 1.59)	1.6E-04	1.56	(1.15, 2.12)	4.0E-03	2.09	(1.42, 3.08)	2.1E-04
IVW MR Effect							-0.38	0.02	9.7E-74	1.35	(1.18, 1.55)	1.1E-05	1.75	(1.35, 2.27)	2.6E-05	2.27	(1.62, 3.16)	1.5E-06

Betas for eBMD are given as SD eBMD per SD decrease in E2. Odds ratios (OR) for fractures are given per SD decrease in E2. Non-vertebral major osteoporotic fractures are defined as wrist, arm, or hip fractures. eBMD = estimated BMD in the heel using ultrasound, IVW = inverse variance weighted.

Figure 1.

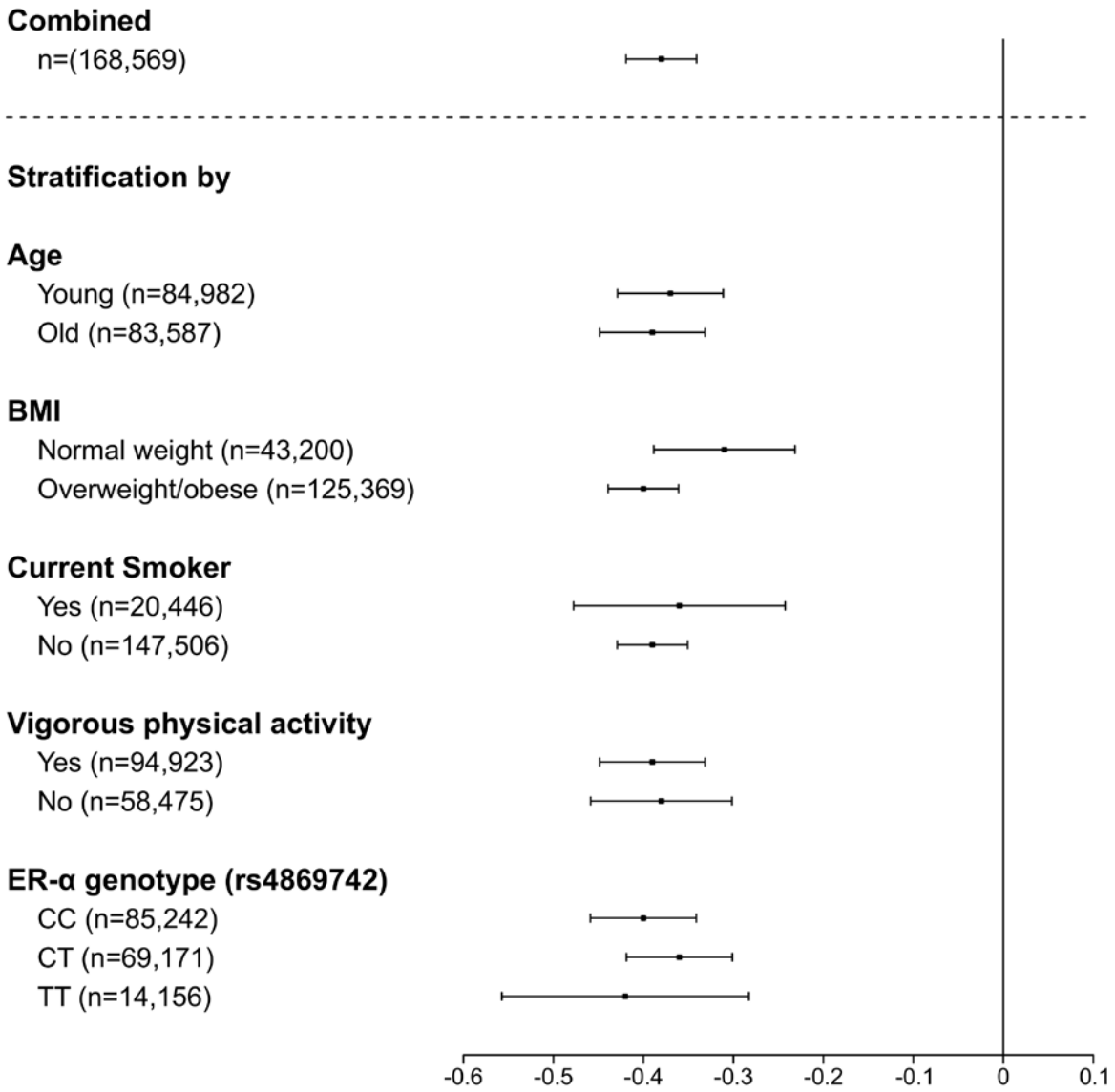

Combined
n=(168,569)
Stratification by
Age
Young (n=84,982)
Old (n=83,587)
BMI
Normal weight (n=43,200)
Overweight/obese (n=125,369)
Current Smoker
Yes (n=20,446)
No (n=147,506)
Vigorous physical activity
Yes (n=94,923)
No (n=58,475)
ER-α genotype (rs4869742)
CC (n=85,242)
CT (n=69,171)
TT (n=14,156)
-0.6
-0.5
-0.4
-0.3
-0.2
-0.1
0
0.1

Figure 2.

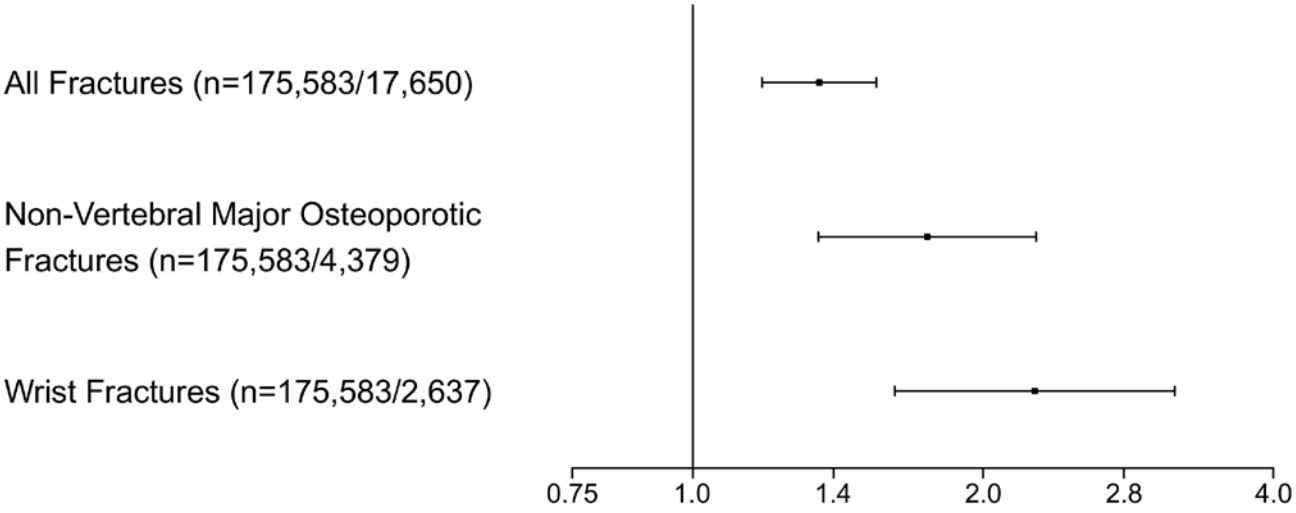
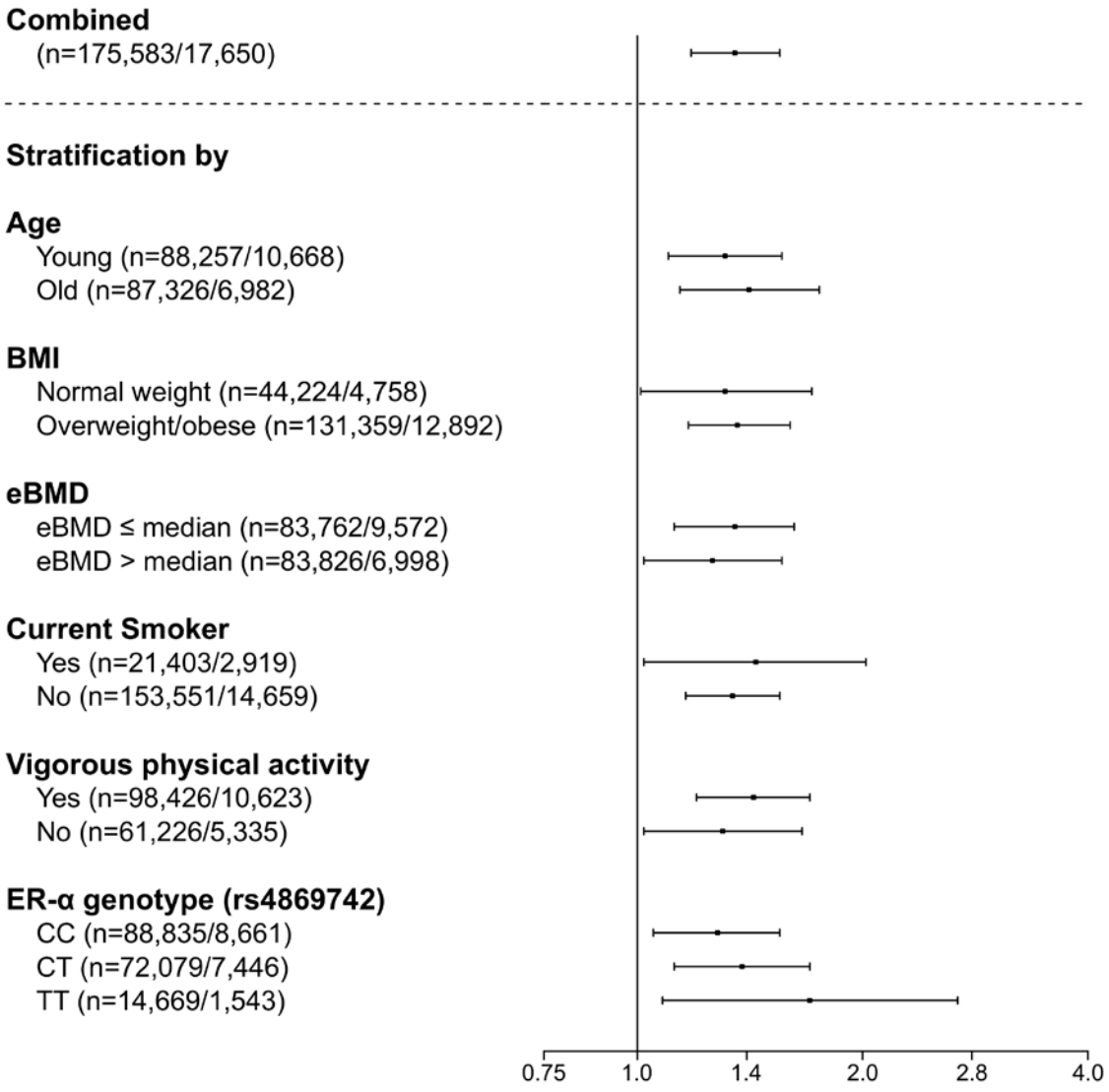

All Fractures (n=175,583/17,650)
Non-Vertebral Major Osteoporotic Fractures (n=175,583/4,379)
Wrist Fractures (n=175,583/2,637)
0.75
1.0
1.4
2.0
2.8
4.0

Figure 3.


Combined
(n=175,583/17,650)
Stratification by
Age
Young (n=88,257/10,668)
Old (n=87,326/6,982)
BMI
Normal weight (n=44,224/4,758)
Overweight/obese (n=131,359/12,892)
eBMD
eBMD ≤ median (n=83,762/9,572)
eBMD > median (n=83,826/6,998)
Current Smoker
Yes (n=21,403/2,919)
No (n=153,551/14,659)
Vigorous physical activity
Yes (n=98,426/10,623)
No (n=61,226/5,335)
ER-α genotype (rs4869742)
CC (n=88,835/8,661)
CT (n=72,079/7,446)
TT (n=14,669/1,543)
0.75
1.0
1.4
2.0
2.8
4.0